

ORIGINAL ARTICLE

Effects of culture conditions on the size, morphology and wet density of spores of *Bacillus cereus* 569 and *Bacillus megaterium* QM B1551

K. Xu Zhou¹, F. Wisnivesky^{2,*}, D.I. Wilson¹ and G. Christie¹

¹ Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK

² Department of Materials Science and Metallurgy, University of Cambridge, Cambridge, UK

Significance and Impact of the Study: Bacterial spores of the genera *Bacillus* and *Clostridium* represent nature's most durable cells in terms of their extreme resistance to a variety of deleterious environments. As a result, they are of concern in the food processing, healthcare and other sectors, and are of increasing biotechnological interest. Improved understanding of variance in spore size, morphology and density may aid the development of certain spore-associated applications (e.g. spore surface display) while contributing to active areas of research such as spore adhesion and resistance to heat.

Keywords

Bacillus, culture conditions, morphology, spore size, wet density.

Correspondence

Graham Christie, Department of Chemical Engineering and Biotechnology, University of Cambridge, Philippa Fawcett Drive, CB3 0AS Cambridge, UK.

E-mail: gc301@cam.ac.uk

*Present address: Florencia Wisnivesky, Page White and Farrer, London, UK.

2017/0222: received 1 February 2017, revised 4 April 2017 and accepted 6 April 2017

doi:10.1111/lam.12745

Abstract

The influence of variable culture conditions on the size and wet density of spores of *Bacillus cereus* and *Bacillus megaterium* were examined in this work. Culture temperature and initial pH was shown to have a significant impact on the size of both species, with increasingly alkaline culture media and elevated culture temperatures resulting in spores that were, on average, up to 25% reduced in volume. Increasing concentrations of inorganic salts in sporulation media exerted differing effects on each species; whereas a fivefold increase in the concentration of all salts resulted in only minor differences to the dimensions of *B. cereus* spores, *B. megaterium* spores became more elongated, displaying an average increase in volume of almost 30%. Similarly, as the spore elongated to yield aspect ratios larger than 1.4, their shape changed from typical prolate spheroids to cylinders with hemispherical ends. In contrast with previous studies, culture conditions employed in this study exerted no discernible impact on the wet density of *B. cereus* or *B. megaterium* spores.

Introduction

Bacterial endospores of the *Bacillales* and *Clostridiales* are formed in response to the depletion of nutrients during vegetative growth. They are metabolically dormant and highly resistant to several forms of environmental stress that would otherwise be lethal to vegetative cells (Driks 2002). Spores are well-placed to spread foodborne diseases and cause spoilage as they combine the ability to survive a variety of harsh environmental conditions with the capacity to adhere to a variety of solid surfaces (Rönner *et al.* 1990). As a result, spore-forming bacteria are of particular concern to the food industry (Brown 2000).

During the process of sporulation, vegetative cells reduce their mass, ultimately preserving essential genetic information and yielding spores that are denser and smaller in size than the parental cell. Analysing spore size and density is of interest to both fundamental (Tisa *et al.* 1982; Leuschner and Lillford 2000) as well as applied (Mercier-Bonin *et al.* 2011) fields of research. The most recent and comprehensive work in this area is perhaps that of Carrera *et al.* (2007), who measured size data for eight different *Bacillus* species. Essentially, they showed that the spore shape may be approximated as a prolate spheroid, with an aspect ratio between 1.2 and 2 and a typical diameter of approximately 0.85 μm . The same

authors also observed significant differences in size amongst different strains of the same species, with the mean length of six different *Bacillus anthracis* strains varying between 1.23 ± 0.08 and 1.67 ± 0.20 μm . In subsequent work, the wet densities of the same spores was found to vary within a narrow range i.e. between 1.150 and 1.223 g cm^{-3} (Carrera *et al.* 2008). By demonstrating a strong negative correlation between spores' wet density and volume, the same authors suggested that water uptake may account for the observed trends. In a related context, Plomp *et al.* (2005) investigated the impact of dehydration on spore size and determined that dehydrated *Bacillus atrophaeus* spores were on average 12% smaller than hydrated spores. However, while there is a wealth of information scattered in the literature that details information on the size and density of various *Bacillus* species, the few studies that have examined the influence of culture conditions on these properties appear to be limited to density (Cazemier *et al.* 2001; Melly *et al.* 2002).

In the current work, the size and morphology of spores of *Bacillus cereus* 569 and *Bacillus megaterium* QM B1551, prepared by nutrient starvation under variable culture conditions, were analysed by scanning electron microscopy (SEM) and by density gradient centrifugation. Spores of the best-studied *Bacillus* species, *Bacillus subtilis*, were examined in some experiments as a comparative benchmark. We anticipate that information pertaining to spore morphology and dimensions may be of applied interest, for example, when attempting to estimate surface contact area and other modelling parameters involving *Bacillus* spores.

Results and discussion

The purpose of the current work was to examine differences in the size, morphology and density of spores of principally *B. cereus* and *B. megaterium* when sporulated by nutrient exhaustion under variable culture conditions (Table S1). The work includes a detailed quantitative analysis of the surface area of the outermost exosporia associated with spores of these species. In our opinion, the SEM technique employed permits a more accurate assessment of the size of these irregularly shaped structures compared to thin-section TEM based analyses reported previously (Carrera *et al.* 2007; Faille *et al.* 2010). As well as being of inherent interest, such information should be of use for applications that utilize mathematical models of spores, for example, when investigating spore adhesion.

Spores of all three species employed in this work possess distinctive morphologies that require the quantification of different features to adequately identify changes

induced by sporulation under variable conditions (Fig. S1). Whereas spores of *B. subtilis* can adequately be described with a length, L , and a diameter, D , the ratio of which yields the aspect ratio for the spore, spores of *B. cereus* and *B. megaterium* are enveloped in an additional exosporium layer. In the case of *B. cereus*, the exosporium is relatively large and of irregular shape; accordingly, these spores were individually analysed to determine the flattened area, A , of this structure. In contrast, the exosporium in *B. megaterium* spores is smaller and closer fitting, presenting itself as a regular shaped lip of width, w , surrounding the central spore structure.

Bacillus cereus and *B. megaterium* spores were prepared by nutrient exhaustion in their respective basal sporulation media (see Table S2) in which either the temperature, initial pH and/or concentration of inorganic salts was modified to assess the impact on the size and morphology of the resultant spores. The dimensions, volume, exosporium surface area, and t -test results of each spore sample are summarized in Table 1. Scanning electron micrographs of spores that are representative of each culture condition are shown in Fig. S2. The distribution and differences in dimensions between the three *Bacillus* species studied can be found in the supporting information (Fig. S3).

Results from the work revealed that significant changes to the average size of spores could be conferred by modifications – in terms of temperature, pH and inorganic salt concentration – to the sporulation conditions. The average volume of the largest *B. megaterium* spore sample, for example, was approximately double that of the smallest spore of the same species ($0.38 \mu\text{m}^3$ vs $0.79 \mu\text{m}^3$). In general, increases to the incubation temperature or alkalinity of the sporulation medium resulted in a significant (P values $\ll 0.05$) reduction (20 to 25%) to the volume of *B. megaterium* and *B. cereus* spores. However, the precise effects of temperature and pH on spore formation have not been established. Presumably the effects are mediated at the genetic regulatory level, insight to which could be gained from transcriptomic and proteomic analyses. Although not monitored through the culture period in this work, the pH of the growth medium, whether buffered or not, does not remain constant during sporulation. Mazas *et al.* (1997), for example, demonstrated that changes in pH during sporulation induced by nutrient exhaustion are particularly pronounced in initially acidic media, increasing from an initial pH 5.5 to pH 6.1 after spore formation in a buffered medium (Mazas *et al.* 1997), and to 7.0 in a weakly buffered medium (Posada-Urbe *et al.* 2015). In contrast, the pH of initially neutral and alkaline solutions remains relatively constant throughout spore formation (Mazas *et al.* 1997). Regardless, it seems that the cellular response to variance in the

Table 1 Summary of measured and calculated features for *Bacillus* spores cultured under different conditions and the outcome of Student's *t*-test to check the significance of observed differences. The values shown are averages from approximately 100 spores sampled from two independent batches of spores, with the standard deviation in brackets. One-micron diameter microspheres were measured as a control sample

Measured features		Calculated features					t-test P-value					
		Spores measured	Length (μm)	Diameter (μm)	Exosporium measure*	Spore volume† (μm ³)	Exosporium surface area‡ (μm ²)	Aspect ratio	P _{Length}	P _{Diameter}	P _{Volume}	P _{Exosporium}
Label												
Microspheres												
1 μm microspheres	100	1.16 (0.09)	1.14 (0.1)	N/A	N/A	N/A	1.02 (0.05)	N/A	N/A	N/A	N/A	N/A
<i>Bacillus megaterium</i>												
BM	123	1.15 (0.15)	0.91 (0.07)	0.15 (0.04)	0.51 (0.11)	4.38 (0.58)	1.26 (0.17)	N/A	N/A	N/A	N/A	N/A
BM25	101	1.17 (0.13)	0.89 (0.08)	0.16 (0.03)	0.49 (0.11)	4.49 (0.49)	1.33 (0.17)	0.29	0.18	0.29	0.12	0.12
BM37	111	1.00 (0.10)	0.84 (0.08)	0.18 (0.04)	0.38 (0.09)	4.06 (0.47)	1.20 (0.15)	0.00	0.00	0.00	0.00	0.00
BMpH9	104	1.13 (0.12)	0.89 (0.08)	0.20 (0.05)	0.48 (0.11)	4.69 (0.58)	1.28 (0.15)	0.05	0.42	0.05	0.00	0.00
BMpH5.5	108	1.08 (0.13)	0.85 (0.08)	0.17 (0.05)	0.41 (0.11)	4.24 (0.65)	1.27 (0.18)	0.00	0.00	0.00	0.09	0.09
BM1	100	1.30 (0.16)	0.90 (0.09)	0.19 (0.05)	0.65 (0.18)	5.08 (0.70)	1.44 (0.17)	0.00	0.00	0.00	0.00	0.00
BM1, 25, pH9	103	1.40 (0.18)	0.96 (0.10)	0.17 (0.04)	0.79 (0.19)	5.32 (0.64)	1.46 (0.20)	0.00	0.00	0.00	0.01	0.01
<i>Bacillus cereus</i>												
BC	103	1.54 (0.11)	0.85 (0.03)	0.66 (0.35)	0.71 (0.09)	5.42 (0.92)	1.81 (0.14)	N/A	N/A	N/A	N/A	N/A
BC25	100	1.51 (0.12)	0.83 (0.06)	0.74 (0.36)	0.67 (0.13)	5.43 (1.05)	1.82 (0.17)	0.08	0.01	0.01	0.98	0.98
BC37	103	1.44 (0.14)	0.78 (0.05)	0.35 (0.24)	0.57 (0.12)	4.25 (0.90)	1.85 (0.16)	0.00	0.00	0.00	0.00	0.00
BCpH9	107	1.53 (0.16)	0.80 (0.05)	0.66 (0.32)	0.65 (0.13)	5.18 (1.00)	1.90 (0.18)	0.52	0.00	0.00	0.05	0.05
BCpH5.5	109	1.39 (0.11)	0.77 (0.04)	0.54 (0.30)	0.53 (0.08)	4.44 (0.82)	1.82 (0.13)	0.00	0.00	0.00	0.00	0.00
BC1	100	1.51 (0.10)	0.81 (0.04)	1.02 (0.36)	0.64 (0.08)	5.86 (0.90)	1.87 (0.15)	0.03	0.00	0.00	0.00	0.00
BC2	100	1.53 (0.08)	0.82 (0.05)	0.47 (0.30)	0.67 (0.09)	4.88 (0.80)	1.88 (0.14)	0.78	0.00	0.00	0.00	0.00
BC2, pH9	126	1.54 (0.12)	0.81 (0.05)	0.52 (0.33)	0.67 (0.14)	5.01 (1.02)	1.90 (0.14)	0.81	0.00	0.00	0.00	0.00
<i>Bacillus subtilis</i>												
BS	150	1.30 (0.11)	0.75 (0.04)	N/A	0.47 (0.07)	N/A	1.73 (0.17)	N/A	N/A	N/A	N/A	N/A

*Exosporium measure defined differently for *B. megaterium* and *B. cereus* spores. In *B. cereus* this refers to the 2D area, in μm^2 , beyond the main spore core and in *B. megaterium* it refers to the width, in μm of the lip surrounding the spore core.

†The spore volume is evaluated using equation (1) in the supporting information except for spores with an aspect ratio of 1.4 or less where equation (2) is used.

‡The exosporium surface area is evaluated using equations (3) and (4) in the supporting information for *B. megaterium* and *B. cereus*, respectively.

pH of the growth medium somehow impacts upon the average size and morphology of the resultant spores. Whether these pH effects are predominantly influenced by the initial pH of the culture medium, or by the pH of the medium upon entry to sporulation, remains to be clarified. Additionally, Mazas *et al.* (1997) observed that *B. cereus* spores grown under alkaline conditions were more resistant to wet heat, which, when coupled with the smaller size of these spores, implies that a reduced core water content is likely to be directly associated with these observations.

Whereas previous work has indicated that increasing the concentration of certain inorganic salts (e.g. MgSO_4) in sporulation media can improve sporulation efficiency (Shi and Zhu 2007; Posada-Urbe *et al.* 2015), results from the present study reveal that increases to the overall concentration of inorganic salts in the medium can significantly influence spore size. *Bacillus megaterium* spores prepared in supplemented nutrient broth (SNB) with increased supplementary salt levels, for example, were shown to adopt a larger and more elongated morphology versus spores prepared in standard SNB medium. This was unexpected since an increase in the osmolarity of the growth medium might be expected to dehydrate and reduce the volume of the spore core. A change in the aspect ratio of *B. megaterium* spores was not observed with spores prepared under conditions of variable temperature and pH, and hence, at least some of the mechanisms that determine spore size and morphology under each of these conditions are likely to be different. Notably, the morphology of *B. cereus* spores prepared in enhanced salt medium was also modified, manifest as an enlarged exosporium. An objective for future work in this area will be to identify the molecular genetic and or biochemical mechanisms that underpin these modifications to the spore morphology.

Having examined the effect of individual parameters on resultant spore morphology, a limited number of combined effects of temperature, pH and inorganic salt concentrations were examined (Table S1). For *B. megaterium* this entailed sporulation at 25°C in SNB supplemented with 5× inorganic salts and pH adjusted to 9.0. *Bacillus cereus* spores were prepared in CCY medium adjusted to pH 9.0 and supplemented with 5× inorganic salt concentration. Essentially, for both species, the resultant spores resembled those prepared in medium containing only enhanced inorganic salt concentrations (Fig. S2). Hence, in the case of *B. megaterium*, the reduction in spore size observed when sporulation occurs at pH 9.0, is not repeated in the presence of 5× salts and the effect of reducing temperature was also shown to be minimal compared to the effect of salt concentration on *B. megaterium* spore formation. In fact, spores produced in the

pH and salt modified medium are longer, suggesting that the latter parameter is dominant in influencing spore morphology. Similarly, increased alkalinity appears to exert minimal effect on *B. cereus* spore morphology in the presence of enhanced inorganic salt concentration, with the resultant spores being more akin to those formed in the presence of enhanced salts at pH 7.2 (*P*-values for all dimensions between 'BC2' and 'BC2pH9' are above 0.45). These results indicate that the concentration of inorganic salts is the dominant influence on spore morphology, at least under the conditions employed in this study. These observations may be considered in the context of the findings of Monteiro *et al.* (2005), who demonstrated that media composition – in terms of nutrient and salt content – influenced sporulation efficiency, whereas changes in pH from 6.0 to 9.0 had no influence on the number of spores formed. Hence, it seems that salts used to supplement sporulation media influence both the efficiency of sporulation and the morphology of the resultant spores.

Having demonstrated that sporulation conditions affect the size and morphology of spores of both *B. cereus* and, in particular, *B. megaterium*, we then sought to determine whether the wet density of the resultant spores was similarly affected. Figure 1 shows that the density of all three *Bacillus* species used in this study, as measured via Percoll density gradient centrifugation, exceeded the average vegetative cell density ($\sim 1.10 \text{ g cm}^{-3}$ (Bratbak and Dundas 1984)). Band 1b observed in the *B. megaterium* sample in Fig. 1 was subsequently revealed by microscopy to comprise germinating spores. Similarly, the opaque region between the two dominant bands was observed to comprise partially germinated spores (in both cases spore germination was triggered by an unknown component of the density measurement medium). Finally, the wet density of spores prepared under variable sporulation conditions were examined in this work (Table 2). A review of the literature reveals a lack of consensus over the densities of *Bacillus* spores, with significant systematic variations being reported that seem to be dependent on the choice of measurement technique (Table S3). One has to consider also that spores subject to density gradient analyses typically settle in wide bands that yield large uncertainties, which combined with parallax errors, renders density estimates that are accurate to only two decimal places. Regardless, in this work, *B. megaterium* spores were observed to have the highest density, followed by *B. subtilis* and then *B. cereus*. Unexpectedly, the densities of *B. megaterium* spores produced under variable conditions were shown to be similar, indicating that the overall water content of these spores were comparable, despite significant differences in spore morphology and size. However, Percoll used in this study does not permeate the spore coat, and therefore provides measurements of the density

of the entire spore (Tisa *et al.* 1982). Future work in this area should therefore consider the use of alternative techniques that measure the density of the spore core, and which may reveal localized differences in density between spores produced under variable conditions. As with spore morphology and size, physicochemical factors such as pH and temperature could feasibly influence patterns and levels of gene expression during sporulation, resulting in the formation of spore populations with variable density distributions.

Materials and methods

Bacillus strains used, spore culture and purification

The three species of *Bacillus* used in this work were *B. megaterium* QM B1551, *B. cereus* 569 and *B. subtilis* 168 (PS832), which are all considered to be lab strains. The sporulation procedures followed were essentially those described by Nicholson and Setlow (1990), Clements and Moir (1998) and Christie *et al.* (2010) for the formation of *B. subtilis*, *B. cereus* and *B. megaterium* spores, respectively. Medium components and their concentrations are provided in the supporting information (Table S2). Essentially, *B. megaterium* and *B. cereus* spores were prepared in 2 l baffled Erlenmeyer flasks containing 300 ml SNB (for *B. megaterium*) or CCY medium (for

Table 2 Wet densities for dormant and germinating *Bacillus* spores

		Wet density (g cm ⁻³)	
Label	Average spore volume (μm ³)	Dormant spores	Germinated spores
<i>Bacillus megaterium</i>			
BM	0.51	1.20 ± 0.02	1.13 ± 0.03
BM25	0.49	1.21 ± 0.01	N/A
BM37	0.38	1.20 ± 0.01	N/A
BMpH5.5	0.48	1.20 ± 0.02	1.11 ± 0.02
BMpH9	0.41	1.21 ± 0.02	1.11 ± 0.02
BM1	0.65	1.20 ± 0.01	N/A
BM1, 25, pH9	0.79	1.21 ± 0.01	1.13 ± 0.01
Other <i>Bacillus</i> species			
<i>Bacillus cereus</i>	0.71	1.14 ± 0.03	N/A
<i>Bacillus subtilis</i>	0.47	1.16 ± 0.04	N/A

B. cereus), agitated at 225 rev min^{-1} at the designated temperature for 72 h. *Bacillus subtilis* spores were prepared on solid 2xSG medium, incubated at 37°C for 5 days. Sporulation media were inoculated with 0.5 ml (flasks) or 0.2 ml (plates) of cells cultured overnight in LB medium. Spores were purified by 6–8 cycles of centrifugation ($15\,000g$ for 10 min) and resuspension in ice-cold deionized water until the suspension was free from vegetative cells and debris (>95% spores confirmed by optical microscopy). *Bacillus cereus* spores were purified

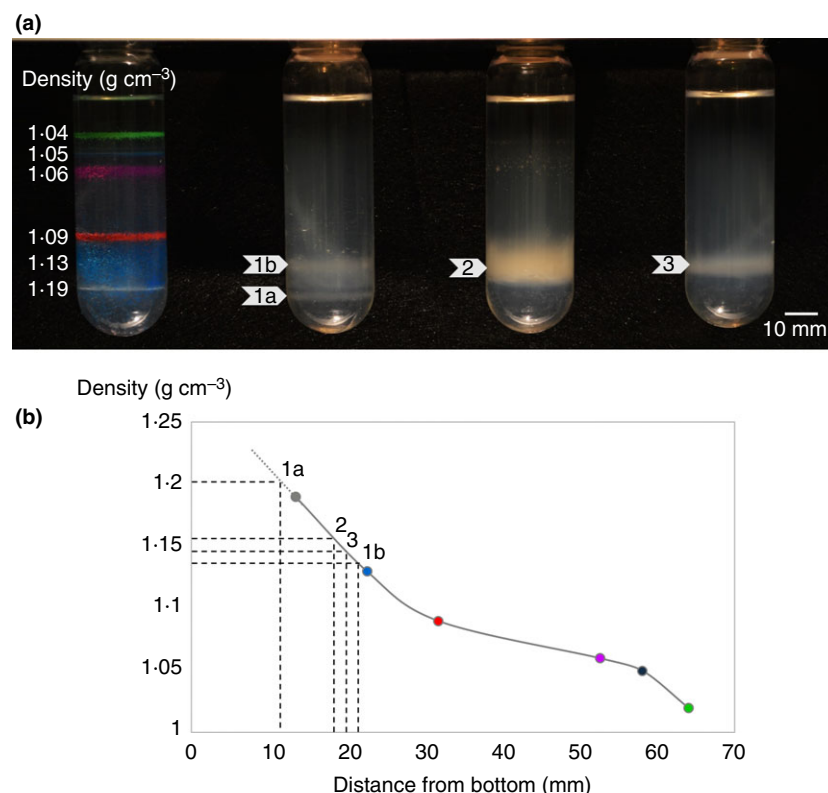


Figure 1 Measuring the wet density of *Bacillus* spores. (a) Centrifuge tubes with Percoll density gradients containing, from left to right: coloured density marker ladder, *Bacillus megaterium* spores [1a] and germinating *B. megaterium* spores [1b], *Bacillus subtilis* spores [2], *Bacillus cereus* spores [3]; (b) plot of density as a function of distance from the bottom of the centrifuge tube. The disperse band marking 1.13 g cm^{-3} was estimated as the midpoint between the adjacent bands. The density of spores higher than 1.19 g cm^{-3} was estimated by linear extrapolation as shown by the dotted grey line. [Colour figure can be viewed at wileyonlinelibrary.com]

in the absence of commonly employed surfactants since our unpublished analyses indicate that such compounds can alter the surface properties of the resultant spores. Purified spores were stored on ice in deionized water at an optical density (at 600 nm) of ~50.

Modified sporulation conditions

Sporulation conditions were modified for *B. cereus* and *B. megaterium* in terms of initial medium pH and inorganic salt concentration in order to assess the impact of these parameters on the resultant spores size, shape and density (Table S1). Spores were also produced at 25, 30 and 37°C to introduce an additional variable parameter. Initial medium adjustments to pH 5.5, 7.2 or 9.0 were made by adding 10 mol l⁻¹ potassium hydroxide or hydrochloric acid as appropriate. Inorganic salt concentration was scaled up for each salt mixture by a factor of 5× and 12.5× with respect to the original (see supporting information). The range of culture conditions and spore sample labels used in this work are detailed in Table S1.

Sample preparation for scanning electron microscopy (SEM)

0.5 ml of spore suspension (OD₆₀₀ ~10) was deposited on a 10 mm diameter glass cover slip. After 5 min, the cover slips were rinsed gently with distilled water to remove any unbound spores and rapidly frozen in order to preserve the spores' morphological features. Frozen samples were then mounted onto carbon adhesive Leit tabs (Agar Scientific, Essex, UK) and lyophilized overnight at a fixed pressure of 10⁻³ mbar, with a temperature profile of -90°C for 2 h followed by incremental 10°C increases in temperature per hour until a final temperature of 30°C was attained. Lyophilized samples were then sputter coated with a thin layer (25–50 nm) of conductive carbon and stored in a dry location, at room temperature, prior to imaging. SEM imaging was undertaken using a FEI Nova NANSEM 450 (FEI Company, Hillsboro, OR, USA) scanning electron microscope.

Spore size and morphology analysis

Scanning electron microscopy images were used to evaluate the shape and size of spores prepared in standard and modified culture conditions. Images were taken at locations where spores were sparsely distributed, wherever possible, such that no two spores were in contact. Furthermore, as *B. megaterium* spores settled into different orientations, only those spores that settled with the exosporium aligning parallel to the plane of the tab matrix were measured. At least 100 spores of each sample were measured to evaluate the distribution and average dimension

parameters. Reported values are measurements derived from two separate batches of spores prepared under the same conditions (i.e. approximately 50 spores from each sample) and which showed no observable variance in size distribution. The shape used to estimate spores' volume and surface areas depended on the aspect ratio i.e. the ratio of the spore's length to its diameter. Spores with an aspect ratio >1.4 were approximated as cylinders with hemispherical ends whereas those with lower aspect ratios were approximated as prolate spheroids.

Wet density measurement

Wet density measurements were performed in Percoll gradients using a procedure similar to that described by Carrera *et al.* (2008). As per the manufacturer's recommendations, the stock Percoll solution was adjusted to a final concentration of 0.15 mol dm⁻³ NaCl and buffered with 20 mmol dm⁻³ Tris-HCl pH 7.5 to yield an iso-osmotic Percoll solution. This solution was then aliquoted into centrifuge tubes containing 25 ml of each solution. One tube contained a density ladder with 150 µl of each density marker bead, polystyrene and PMMA microspheres and approximately 200 µl of a 5 wt.% spore suspension was added to the other tubes. Density gradients were then achieved by centrifuging these tubes in a Thermo Fisher Scientific (Paisley, UK) F20-12 × 50 LEX fixed angle rotor at 40 000 g for 1 h. After centrifugation, triplicate images of each set of tubes were taken using a Nikon D3200 digital camera (Nikon UK, Kingston upon Thames, UK). In order to evaluate the spore's wet density, the position of each band relative to the density ladder was computed on ImageJ (NIH, Bethesda, MD) using the average of the three images.

Statistical analysis

Statistical analyses were performed in Microsoft Excel (Redmond, WA). Spore length, diameter, volume and exosporium surface area were tested for the null hypothesis using a two-tailed heteroscedastic Student's *t*-test. In general, the null hypothesis is rejected if the probability of obtaining an equal result is below 5% (Nuzzo 2014) and the two samples are considered to be significantly different. The errors for dimensions quoted in text represent 95% confidence interval estimates of the mean.

Acknowledgements

Support of an EPSRC DTG studentship for KXZ and the assistance of Dr Nan Li and Dr Fernando da Cruz Vasconcellos with the acquisition of SEM images are gratefully acknowledged.

Conflict of Interest

The authors declare no conflicts of interest.

References

- Bratbak, G. and Dundas, I. (1984) Bacterial dry matter content and biomass estimations. *Appl Environ Microbiol* **48**, 755–757.
- Brown, K.L. (2000) Control of bacterial spores. *Br Med Bull* **56**, 158–171.
- Carrera, M., Zandomeni, R.O., Fitzgibbon, J. and Sagripanti, J.L. (2007) Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. *J Appl Microbiol* **102**, 303–312.
- Carrera, M., Zandomeni, R.O. and Sagripanti, J.L. (2008) Wet and dry density of *Bacillus anthracis* and other *Bacillus* species. *J Appl Microbiol* **105**, 68–77.
- Cazemier, A.E., Wagenaars, S.F. and ter Steeg, P.F. (2001) Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. *J Appl Microbiol* **90**, 761–770.
- Christie, G., Ustok, F.I., Lu, Q., Packman, L.C. and Lowe, C.R. (2010) Mutational analysis of *Bacillus megaterium* QM B1551 cortex-lytic enzymes. *J Bacteriol* **192**, 5378–5389.
- Clements, M.O. and Moir, A. (1998) Role of the gerI operon of *Bacillus cereus* 569 in the response of spores to germinants. *J Bacteriol* **180**, 6729–6735.
- Driks, A. (2002) Maximum shields: the assembly and function of the bacterial spore coat. *Trends Microbiol* **10**, 251–254.
- Faille, C., Lequette, Y., Ronse, A., Slomianny, C., Garenaux, E. and Guerardel, Y. (2010) Morphology and physico-chemical properties of *Bacillus* spores surrounded or not with an exosporium: consequences on their ability to adhere to stainless steel. *Int J Food Microbiol* **143**, 125–135.
- Leuschner, R.G. and Lillford, P.J. (2000) Effects of hydration on molecular mobility in phase-bright *Bacillus subtilis* spores. *Microbiology (Reading, England)* **146**(Pt 1), 49–55.
- Mazas, M., Lopez, M., Gonzalez, I., Bernardo, A. and Martin, R. (1997) Effects of sporulation pH on the heat resistance and the sporulation of *Bacillus cereus*. *Lett Appl Microbiol* **25**, 331–334.
- Melly, E., Genest, P.C., Gilmore, M.E., Little, S., Popham, D.L., Driks, A. and Setlow, P. (2002) Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. *J Appl Microbiol* **92**, 1105–1115.
- Mercier-Bonin, M., Dehouche, A., Morchain, J. and Schmitz, P. (2011) Orientation and detachment dynamics of *Bacillus* spores from stainless steel under controlled shear flow: modelling of the adhesion force. *Int J Food Microbiol* **146**, 182–191.
- Monteiro, S.M., Clemente, J.J., Henriques, A.O., Gomes, R.J., Carrondo, M.J. and Cunha, A.E. (2005) A procedure for high-yield spore production by *Bacillus subtilis*. *Biotechnol Prog* **21**, 1026–1031.
- Nicholson, W.L. and Setlow, P. (1990) Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus*, ed. Harwood, C.R. and Cutting, S.M. pp. 391–450. Chichester, UK: John Wiley and Sons.
- Nuzzo, R. (2014) Scientific method: statistical errors. *Nature* **506**, 150–152.
- Plomp, M., Leighton, T.J., Wheeler, K.E. and Malkin, A.J. (2005) The high-resolution architecture and structural dynamics of *Bacillus* spores. *Biophys J* **88**, 603–608.
- Posada-Urbe, L.F., Romero-Tabarez, M. and Villegas-Escobar, V. (2015) Effect of medium components and culture conditions in *Bacillus subtilis* EA-CB0575 spore production. *Bioprocess Biosyst Eng* **38**, 1879–1888.
- Rönnner, U., Husmark, U. and Henriksson, A. (1990) Adhesion of *Bacillus* spores in relation to hydrophobicity. *J Appl Bacteriol* **69**, 550–556.
- Shi, F. and Zhu, Y. (2007) Application of statistically-based experimental designs in medium optimization for spore production of *Bacillus subtilis* from distillery effluent. *Biocontrol* **52**, 845–853.
- Tisa, L.S., Koshikawa, T. and Gerhardt, P. (1982) Wet and dry bacterial spore densities determined by buoyant sedimentation. *Appl Environ Microbiol* **43**, 1307–1310.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic illustration of characteristic (a) *Bacillus megaterium* spores; (b) *Bacillus cereus* spores and (c) *Bacillus subtilis* spores. Dimensions: L and D are the spore length and diameter, respectively; w is the exosporium lip width in *B. megaterium* spores and A is the additional area covered by the exosporium beyond the *B. cereus* spore coat.

Figure S2. SEM images showing the appearance of *Bacillus cereus* and *Bacillus megaterium* spores cultured under: (a) standard conditions; (b) variable temperatures; (c) variable initial pH; (d) increased inorganic salt concentrations; (e) modified pH, temperature and salt concentration. The scale in (b) – (e) is the same as in (a) for each species.

Figure S3. SEM images and histograms showing the distribution of lengths and diameters of (a) *Bacillus cereus* spores; (b) *Bacillus megaterium* spores and (c) *Bacillus subtilis* spores. The bars on the SEM images represent 1 μm . Lines on the histograms show the cumulative distribution function.

Table S1. Spore culture conditions and their corresponding labels.

Table S2. Composition of basal sporulation media.

Table S3. Comparison of the wet densities of dormant *Bacillus* spores reported in literature obtained using various measurement methods.